Antimicrobial activity of probiotic Lactobacillus strains towards Salmonella enterica ser enteritidis in whey

N Goyal, K Dixit, D Gandhi

Citation

N Goyal, K Dixit, D Gandhi. *Antimicrobial activity of probiotic Lactobacillus strains towards Salmonella enterica ser enteritidis in whey*. The Internet Journal of Microbiology. 2007 Volume 5 Number 1.

Abstract

Aim: The aim of this study therefore, was to evaluate the suitability of whey as a delivery agent of probiotics culture towards diarrhoea causing pathogens.

Procedure: Seven Lactobacillus strains (from NCDC and MTCC), including DDS-1 (well established probiotic culture) were screened, under controlled condition, for their capacity to inhibit Salmonella enteritidis in fermented whey.

Result: The inhibitory mechanism was found to be dependent on the lowering of the pH of the medium and production of lactic acid. The antibacterial activity of L. paracasei 17 and L. casei 299 from NCDC were more effective in inhibiting Salmonella enteritidis, within 8hrs contact of fermented whey.

Conclusion: Fermented whey can be use for the management against enteric diarrhoea

INTRODUCTION

Past decades have witnessed the applications of probiotics in the prevention and management of gastrointestinal disorders (Heyman and Menard, 2002).These microbes are designed to improve intestinal microbial balance and partake in normal bacterial-epithelial crosstalk and offer a potential promising approach to the management of intestinal problems caused by stress (Servin, 2004).

Different probiotic species and even different strains within a species exhibit distinctive properties that can markedly affect their survival in foods, fermentation characteristics and other probiotic properties. Among various species, Lactobacillus acidophilus (Gandhi and Nambudripad, 1978), L. rhamnosus (Isolauri et al, 1995), L. casei, have been shown their efficacy in prophylactic management of acute diarrhoea in children, with an associated increase in the immunity.

Barely few years ago, whey was merely a by-product of cheese and paneer industry, which was usually dumped because it had no value; a practice that was increasingly frowned upon by environmentalists. .Available literature on application of whey in controlling gastrointestinal disorders is not ample. The lactic acid bacteria possess the ability of lactose utilization and hence can form a better resource for maximum lactose bioconversion in whey (Gupta and Gandhi, 1995). The aim of the experiment was to study the ability of Lactobacillus strains to exert antagonistic activity towards Salmonella enteritidis, a potential pathogen causing diarrhoea using simple whey to utilize it as a medium for the delivery of probiotic culture.

MATERIAL AND METHODS

MAINTENANCE AND PROPAGATION OF CULTURES

Six Lactobacillus strains, including DDS-19 (Department of Dairy Science, University of Nebraska) were used in this study and shown in Table 1 along with their source. Working cultures were maintained in MRS broth (de Man et al., 1960) at 37 ^{o} C and sub cultured twice prior to assay. Salmonella enteritidis 3219, an enteric pathogen was obtained from the MTCC, IMTECH and cultured in Brain Heart Infusion (BHI) broth.

LACTIC ACID ESTIMATION IN CELL FREE SUPERNATANT

The concentrations of D-L Lactic acid in cell free

supernatant obtained from MRS and whey of selected cultures was determined using commercial enzymatic kit (Test Combination D-Lactic acid / L-lactic acid UV method; Boehringer Mannheim Gmbh, Germany).

SAMPLE PREPARATION

The sample preparation is required for the colored, acidic, fermented samples of MRS and whey. Accurately weigh approx. 1 to 10 ml of cell free supernatant into 100ml volumetric flask. Add 50ml of warm water. For clarification, add 5ml Carrrez-I solution (3.60g potassium hexacyanoferrate II), 5ml Carrrez II solution (7.2 g zinc sulphate) and 10ml (0.1M) NaOH and 1 ml of filtrate was used for lactic acid assay.

KILLING ASSAY FOR ANTI ACTIVITY

The inhibitory activity of fermented whey CFCS of probiotic cultures towards Salmonella enteritidis was determined by in vitro killing assay. A mid logarithmic phase cells were obtained by inoculating 10 ml LB broth with 100ul of fresh culture and incubated at 37 $^{\circ}$ C for 4h. The Salmonella strain was centrifuged at 5,500x g for 5 min at 4 $^{\circ}$ C and the pellet was washed once with phosphate buffer saline (PBS) and resuspended in PBS. The bacterial cells were counted, and a volume containing 2x10 ^{8} cfu/ml was used to determine the killing activity. A colony count assay was performed by incubating 500µl of Salmonella enteritidis at 2x10 ^{8} in LB medium with 500µl of CFCS (Fermented whey), MRS broth or MRS-LA (pH 4.0-4.4) at 37 $^{\circ}$ C. At predominant interval, up to 8 hrs, aliquots were removed, serially diluted and plated on trypticase soy agar (TSA) to determine the surviving Salmonella.

STATISTICAL ANALYSIS

The experimental data are presented as the mean (SEM). General Linear Model (GLM) procedure with post hoc test was applied to determine whether significant differences existed among different cultures using SYSTAT 6.0.1 Statistical Software Package, 1996, SPSS, Inc., USA. The predetermined acceptable level of probability was 5% ($P < 0.005$) for all comparisons

RESULTS

ANALYSIS OF LACTIC ACID CONCENTRATION OF SELECTED CULTURES

Lactic acid concentration was determined by Boehringer Mannheim D-L Lactic acid kit in the selected best probiotic cultures. After culturing for 24 $^{\circ}$ C at 37 $^{\circ}$ C in MRS broth (containing glucose) as shown in Fig 1 and whey (without any nutrients) as shown in Table 1, lactic acid concentration in cell free supernatant (CFS) was determined.

D-L isomers of lactic acid was estimated in all the seven whey samples inoculated with seven probiotic lactic cultures after an incubation period of 24 hrs. Lactic acid concentration in whey reaches maximally up to 29mM (Table 1) for NCDC 291 followed by NCDC 299 and NCDC 17. The low lactic acid concentration resulted in lesser diffusion in agar well method. Moreover, the findings showed that only NCDC 17 produces more of D-lactic acid (7.2mM/l) as compared to other probiotic strains in fermented whey. Low lactic acid concentration was shown by strains DDS-1 and MTCC 1408.

Figure 1

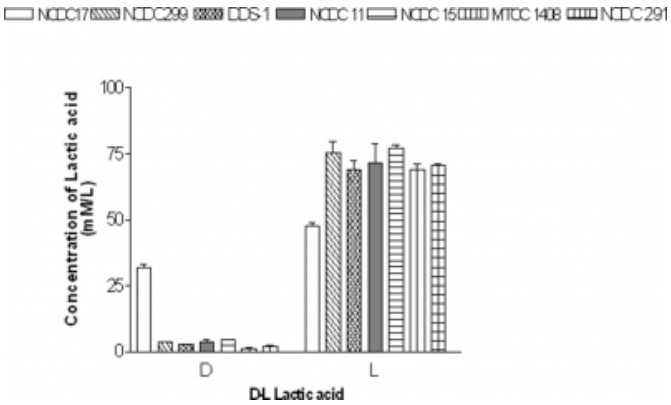
Table 1: D and L lactic acid concentration in fermented whey of seven cultures

S No.	Cultures	Lactic acid (Whey) (mM/l)	
		D	L
1	NCDC 11 *L.acidophilus*	2	23.0
2	NCDC 15 *L.acidophilus*	2.12	19.6
3	NCDC 291 *L.acidophilus*	1.76	28.9
4	NCDC 17 *L.paracasei*	7.2	22.3
5	MTCC 1408 *L.rhamnosus*	2.24	16.7
6	NCDC 299 *L.casei*	1.66	27.9
7	DDS-1 *L.acidophilus*	2.78	14.5

All selected cultures produced L- lactic acid during MRS fermentation in the range of 60Mm – 80mM with a little amount of D-lactic acid. Only L. paracasei NCDC 17 produces both D and L lactic in approximately equal

amounts similar to lactic concentration in whey. NCDC 299 produces maximum L- lactic concentration (79mM/L) trailed by NCDC 15 (78mM/L).

Figure 2



TIME DEPENDENT INHIBITORY OR KILLING ACTIVITY OF FERMENTED WHEY

In vitro killing activity of CFCS was determined against the enteric pathogen up to 8h at predominant intervals, on trypticase soy agar (TSA) to determine the viable bacterial count. In vitro killing activity against Salmonella enteritidis MTCC3219, the most stringent pathogen was determined in cell- free whey supernatant at 37 ^{o} C for 24hrs. In this study, MRS-LA and MRS-HCL were used as positive controls. The reduced viability of the pathogen with the effect of cell free supernatant (Whey and MRS) increases with contact time which can be noticeable from the least square mean (Table 2).

The findings indicated that fermentation of whey with seven Lactobacillus strains followed similar patterns of inhibition i.e. decrease of viable count with increase in time against Salmonella enteritidis. LSD procedure was adopted for pair wise comparison of the means. Low least square mean of NCDC17 and NCDC 299 indicated the low viability of Salmonella enteritidis during the entire experiment. A 5 log reduction in viability of Salmonella was observed within 8hrs of contact by cell free supernatant (whey) of NCDC 17 and NCDC 299. MRS- LA results in a complete death of pathogen with an incubation of 8 hrs irrespective of the strain with an exception of DDS-1. The strain was the least effective in killing the pathogen as specified by its high LSQ (7.160± 0.191).The low mean value (5.139± 0.161) of MRS-CFCS was due to better inhibition than whey CFCS which in turn could produce killing activity than MRS-HCL. 4 th hr and 6 th hr of incubation were the major principal time which could bring out a considerable variability in Salmonella enteritidis count. With an increase in incubation time, more Salmonella cells were killed (Mean: 5.491±0.144) and there is a complete death of pathogen with an incubation of 8 hrs using MRS-LA, irrespective of the strain. In vitro killing activity was cell-free supernatant (CFCS) and time dependent, as observed by the high F value i.e. 33.199 and 83.412., significant at P< 0.001 respectively. The level of inhibitory activity was not strain dependent (p> 0.001).The difference between the antibacterial activity of the CFCS (whey) and acid control samples was also significant (P< 0.001).

Figure 3

Table 2: Least square means of log count of in different CFCS at different time interval

Effect	N	LSQ±S.E
Overall mean	140	6.763±0.72
Strain		
NCDC 11,	20	6.792± 0.196^{a}
NCDC 291	20	6.880± 0.191^{a}
NCDC 17,	20	6.446± 0.191^{a}
NCDC 15	20	6.681± 0.191^{a}
NCDC 299	20	6.659± 0.191^{a}
MTCC 1408	20	6.717±0.196^{a}
DDS-1.	20	7.160± 0.191^{a}
CFCS		
1(MRS LA)	35	5.139± 0.161 ^{a}
2 (Whey-LA)	35	6.395±0.161 ^{b}
3(MRS –HCL)	35	7.193±0.161^{c}
4(Control)	35	8.327±0.161^{d}
Time		
1(0hr)	28	7.898±0.149^{a}
2(2hr)	28	7.319± 0.144^{ab}
3(4hr)	28	6.940±0.144 ^{b}
4(6hr)	28	6.169± 0.144 ^{c}
5(8 hr)	28	5.491±0.144 ^{c}

** Mean with no common superscripts is significantly different (p< 0.001).*

DISCUSSION

With respect to the production of enzyme in MRS, whey due to lack of specific nutrients is not a good medium for β-galactosidase enzymatic activity. It is based on simple conclusion that MRS medium is composed of rich nutrient source as compared to whey and thus lactic cultures grow better in glucose based medium.

It is noteworthy that L-lactic acid, displays a greater antibacterial activity than D-lactic acid in the CFCSs (Fayoul-Messaoudi et al., 2005).But NCDC 17 displayed the maximum antibacterial activity and this could not be attributed to lactic acid alone, as the CFCs displayed killing activity beyond the activity of lactic acid concentration presents (Makras et al., 2006). The antagonistic activity observed against various indicator organisms may be because of cumulative effects of all the antimicrobial

metabolites like lactic acid, acetic acid and oxalic acid secreted by test cultures into the medium (Gilliland and Speck, 1977) that get concentrated and diffuse around the test culture.

High anti bactericidal activity of MRS as compared to whey is due to higher nutritional property of selection medium which affects the production of antimicrobial isolates by Lactobacillus spp. It can be predicted that with the prolongation of incubation time, there is a decrease in pathogen count. Salmonella enteritidis was sensitive to the bactericidal activity of lactic acid produced from probiotic strain and the killing activity started at a lactic acid concentration of 20mM and was complete at 40mM lactic acid. It was found that the killing activities of the whey CFCS obtained after 24hr fermentation decreases the log count significantly. Whereas acidity as adjusted by hydrochloric acid also brought about effects of outer membrane disruption, the direct effect of lactic acid was always stronger than that observed in HCl treated bacteria at the same pH (Alakomi et al., 2000).

The major weapons to combat the enteric enemies, a property endorse by fermented whey are the organic acids. Short chain fatty acid (SCFA), such as acetic acid, citric acid and lactic acid which are the major metabolites of lactic acid bacteria have been reported to be responsible for their antimicrobial activity against pathogens in intestine. Among all the organic acids, lactic acid is the best in inhibiting the proliferation of bacteria that causes putrefaction.

.Lactobacillus strains, a major constituent of fermented whey are mainly saccharolytic, resulting in short chain fatty acid (SCFA) production from lactose. When pH of the environment falls, the concentration of molecular non dissociated form of weak organic acids (VFA) elevates. It is suggested that the mechanism of injury can be divided into two phases i.e. the bacteriostatic effect (between 3.2mM and 62mM) and the bactericidal phase (> 62mM) (Ogawa et al., 2001). This explains well the difference of antimicrobial activity between the effective strains and less effective strains of Lactobacillus. The fermented whey can be effective in indulging in static action against pathogens.

The mechanism involved, is that the undissociated form of the organic acid enters the bacterial cell and dissociates inside the cytoplasm. The eventual lowering of the intracellular pH or the intercellular accumulation of the ionized form of the organic acid, lead to the death of the pathogen (Makras and De Vyust, 2006).Complete inhibition of growth occurred consistently at approximately 10mM undissociated lactic acid for total lactic acid concentrations of 25mM to 100mM (Presser et al., 1997). Consignado et al., 1993 did an in vitro experiment in which successful results of antibacterial activity of L.casei (from Yakult) were obtained against four common diarrhoea causing pathogens i.e. E.coli, Shigella dysenteriae, Salmonella enteritidis and V. cholerae.

The above in vitro study connote that both NCDC 17 and NCDC 299 produced higher lactic acid concentration and can be use for the treatment during diarrhoea caused by Salmonella using whey as a medium.

References

r-0. Alakomi, H.L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latya-kala, K. and Helander, I.M. 2000. Lactic acid permeabilizes gram negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol., 66: 2001-2005.
r-1. Consignado, G.O., Pena, A.C. and Jacalne, A.V. 1993. In vitro sudy on the antibacterial activity of Lactobacillus casei against four diarrhoea causing pathogens. Phil. Microbiol. Infect. Dis., 22(2): 50-55.
r-2. de Man, J.C., Rogosa, M. and Sharpe, M.E. 1960.A medium for cultivation of lactobacilli. J. Appl. Microbiol., 23(1): 130-135.
r-3. Fayol- Messaoudi, D., Berger,C.N., Coconnier-Polter, M.H., Moal,V.L and A.L. Servin. 2005. pH-, Lactic Acid-, and non-Lactic Acid- Dependent activities of Probiotic lactobacilli against Salmonella enterica serovar Typhimurium. Appl. Environ. Microbiol., 71: 6008-6013.
r-4. Gandhi,D.N and Nambudripad, V.K.1978.Implantation of Lactobacillus acidophilus in the intestine of adults suffering from gastrointestinal disorders. XX International Dairy Congress. Brief communication Vol E-1 pp- 972.
r-5. Gilliand, S.E. and Speck, M.L. 1997. Deconjugation of bile acids by intestinal lactobacilli. J. Appl. Bacteriol., 79: 292-301.
r-6. Gupta, R and Gandhi, D.N. 1995. Effect of some supplementation of some nutrients in whey on the production of lactic acid. Indian J. Dairy Sci., 48: 636-641.
r-7. Heyman, M. and Menard, S. 2002. Probiotic microorganisms: how they affect intestinal pathophysiology. Cell. Mol. Life Sci., 59: 1-15.
r-8. Isolauri, E., Joensuu, J., Suomalainen, H., Luomala, M and Vesikari, T. 1995. Improved immunogenicity of oral DxRRV resorting rotavirus vaccine by Lactobacillus casei GG. Vaccine, 13: 310–312
r-9. Makras,L. and de Vyust, L. 2006.The invitro inhibition of gram negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. Int. Dairy J., 16:1049-1057.
r-10. Ogawa, M., Shimizu, K., Nomoto, K., Tanaka, R., Hamabata, T., Yamasaki, S., Takeda, T. and Takeda, Y. 2001 (b). Inhibition of in vitro growth of Shiga toxin-producing E.coli O157:H7 by probiotic Lactobacillus strains due to production of lactic acid. Int. J. Food Microbiol., 68:135-140.
r-11. Presser, K.A., Ratkowsky, D.A. and Ross, T. 1997.

Modelling the growth rate of E.coli as a function of pH and lactic acid Concentration. Appl. Environ. Micobio., 63:2355-2630.

r-12. Servin, A.L.2004. Antagonastic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev., 28: 405-440.

Author Information

Nupar Goyal, PhD
Division of Dairy Microbiology, National Dairy research Institute

Kalpana Dixit
Research Scholar, Division of Dairy Microbiology, National Dairy research Institute

D.N. Gandhi, Ph.D.
Principal scientist, Division of Dairy Microbiology, National Dairy research Institute